1-1-2014

Study of the Mechanisms of Heat Tolerance in Ivy Geraniums

Mingshu Zhang

Study of the mechanisms of heat tolerance in ivy geraniums

By

Mingshu Zhang

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy
in Horticulture
in the Department of Plant and Soil Sciences

Mississippi State, Mississippi

December 2014

Study of the mechanisms of heat tolerance in ivy geraniums

By

Mingshu Zhang

Approved:

_____

Richard L. Harkess
(Co-Major Professor)

_____

Guihong Bi
(Co-Major Professor)

_____

Mengmeng Gu
(Committee Member)

_____

Frank B. Matta
(Committee Member)

_____

Michael S. Cox
(Committee Member/ Graduate Coordinator)

_____

J. Mike Phillips
Department Head

_____

George M. Hopper
Dean)
College of Agriculture and Life Sciences

Name: Mingshu Zhang

Date of Degree: December 13, 2014

Institution: Mississippi State University

Major Field: Horticulture

Major Professors: Richard L. Harkess, Guihong Bi

Title of Study: Study of the mechanisms of heat tolerance in ivy geraniums

Pages in Study: 49

Candidate for Doctor of Philosophy

Ivy geranium (*Pelargonium peltatum*) is a heat susceptible species with its heat tolerance varying among varieties. Reactive oxygen species (ROS) and in-vivo defense systems are related to plant heat damage and heat tolerance. Application of chelated-iron has also been reported to enhance ivy geranium heat tolerance; however, the correlation of ROS, relative enzyme stability, and iron content to differences in heat tolerance in ivy geraniums is unknown. Here we show that the H_2O_2 content and ROS scavenging enzyme stability in ivy geranium varies with varieties and active iron is not related to heat tolerance in ivy geranium. H_2O_2 content in mature leaves in both heat tolerant 'Beach' and sensitive 'Butterfly' increased under heat stress, but 'Butterfly' had a relatively greater increase of this toxic compound. Catalase (CAT) activities in young leaves in both varieties decreased. In young leaves of 'Butterfly', CAT activities decreased to a level significantly lower than that in old leaves while this did not occur in 'Beach'. Superoxide dismutase (SOD) activities in 'Butterfly' young leaves were also decreased. All these phenomenon coincided with the heat tolerance differences of the two varieties. Active iron content only changed with leaf age and did not vary between varieties or

treatments. Our results demonstrated that ROS scavenging ability and relative enzyme stability may indicate heat tolerance in ivy geranium and that iron deficiency was not the cause of heat damage.

Cell Membrane Themostability (CMT) and Triphenyl Tetrazolium Chloride (TTC) cell viability tests are alternative, laboratory-based screening methods for screening for heat-tolerance. Both CMT and TTC tests can represent the variance in heat tolerance observed in ivy geraniums. The results of both CMT and TTC tests correlated well with plant width and growth indexes although their correlations to plant chlorosis were low. Unlike TTC, CMT strongly correlated with plant width. CMT and TTC tests are complementary laboratory-based methods that can be applied to cultivar screening for heat tolerance in ivy geraniums.

DEDICATION

This dissertation is dedicated to my family.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Richard Harkess, my major advisor, for his support to my research for years. Dr. Harkess is a very nice person, actually the nicest I have met. I have learnt not only knowledge, science, and horticulture from him, but also the philosophy of being a good person. I express my deepest thanks to Dr. Harkess.

Second, I am indebted to Dr. Guihong Bi, my co-major advisor. She has provided me very useful and detailed advice on my research. Although I could not see her every day, I did learn a lot from her each time I talked to her.

Third, I am also very thankful to my research committee members Drs. Mengmeng Gu, Frank Matta, and Michael Cox. Dr. Gu invited me to participate a lot in her research projects. The idea about apply CMT and TTC tests on ivy geranium was developed through a project I collaborated with her. I shall also thank Drs. Matta and Cox for serving as my committee members, without which I could not have defended my dissertation.

Next, I also need to thank MSU staff and my schoolmates as horticulture labs always need a lot of cooperations. I appreciate their kind help during my research.

Last but not least, I will thank my families. Without their encouragement, I shall not have finished my dissertation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Ivy geraniums (*Pelargonium peltatum* L.) are heat susceptible and their heat tolerances vary between varieties. Heat stress can cause foliar bleaching, chlorosis and reduced flowering of ivy geranium. These damages reduced the growth and value of ivy geranium products. In this research, the mechanism of heat tolerance of ivy geranium and heat tolerance evaluation methods were both studied. The purpose of this research is to understand the key factors that affect the heat tolerance of ivy geraniums and to develop an accurate and cost efficient variety selection method to aid in breeding heat tolerant varieties.

In current heat tolerance research, reactive oxygen species (ROS) and in vivo defense systems were found to be related to plant heat tolerance. This dissertation focused on the relationship between the ROS scavenging system and ivy geranium heat tolerance. Besides the ROS scavenging system, the effect of specific nutrients, such as iron, on ivy geranium heat tolerance was also studied.

Cultivar screening for heat-tolerant varieties of ivy geranium relies heavily on whole-plant methods. Those methods are time and labor consuming. Laboratory-based screening methods such as cell membrane thermostability (CMT) and Triphenyl Tetrazolium Chloride (TTC) cell viability tests have been introduced as tools in plant breeding. They have been applied to screen for heat tolerance in crops such as wheat and

soybean. Compared to whole-plant methods, these methods are more efficient and cost effective. The possibility to apply these methods to determine ivy geranium heat tolerance was studied.

The objectives of this research were to determine the change in active iron content and hydrogen peroxide (H_2O_2) content in leaves of ivy geranium varieties of different age under heat stress; to determine the relationship of changes in catalase (CAT) and superoxide dismutase (SOD) activities in two varieties of ivy geranium which differ in heat tolerance and the roles of CAT and SOD in ivy geranium heat tolerance; and to determine the extent CMT and TTC assays may be used for selecting heat-tolerance in ivy geranium cultivars and compare the two assays for estimating variability in ivy geranium cultivars for heat tolerance.

CHAPTER II

LITERATURE REVIEW

2.1 Plant Response to Heat Stress

2.1.1 General Introduction

As greenhouse gas emission increases due to the increase in human activities, the global climate is warming. In semi-tropical and tropical regions, the temperature will exceed the optimal conditions for the growth of crops in the near future. Therefore, agronomic crops may suffer more heat damage and the production costs of the agriculture industry will increase. Currently heat stress response and heat tolerance in plants is a hot topic in research.

Heat stress can cause anatomical and physiological changes in plants (Wahid et al., 2007). Under heat stress, the leaf senescence process will be triggered in heat susceptible species (Farooq et al., 2011). Decrease of chlorophyll content was also observed (Todorov et al., 2003). The reproductive organs are also very sensitive to heat stress (Reddy and Kakani, 2007). High temperature during the reproductive phase can cause pollen sterility and decrease of yield (Wassmann et al., 2009). Current researches have been trying to explain the mechanisms of changes caused by heat stress on physiological, protein and molecular levels.

Plant physiological mechanisms related to heat damage and heat tolerance are very complicated. Heat stress triggers the generation of reactive oxygen species and

diminished the activities of phosphoenolpyruvate carboxylase (PEPCase), nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME), fructose-2,6-bisphosphatase (FBPase), pyruvate, phosphate dikinase (PPDK), ribulose bisphosphate carboxylase/oxygenase (Rubicsco), etc. (Sharkey, 2005; Wahid et al., 2007)**.** ROS plays a dual role in this process. ROS can trigger the heat resistant mechanism in the plant while accumulation of ROS might cause further damage to the plant membranes system (Sharkey, 2005). The damage of the membranes system will cause the degradation of enzymes and other proteins. Finally, a decrease of photosynthesis and carboxylation will be observed under heat stress**.**

Changes of the amount of protein were also studied. Xu and Huang's study (2008) shows some of the proteins were down regulated while others were up regulated. Heat tolerant varieties tend to have more up regulated proteins compared to heat sensitive varieties. In their research, they also concluded that the up-regulation of sucrose synthase, glutathione S-transferase (GST), SOD, and heat shock protein (HSP) stress-inducible protein may contribute to the root thermotolerance of a heat tolerant *Agrostis* variety.

Genes related to heat stress were also studied. Currently, most studies focused on the HSP genes. HSP is induced by a sudden high temperature stress. These proteins aid in heat resistance of plants. They act as molecular chaperones which protect other proteins and keep them in stable structures (Wahid et al., 2007). HSP genes have been introduced in many plants such as *Arabidopsis* to increase the heat tolerance of the transgenic plants.

Heat tolerance varies among different plant species. At the onset of heat stress, gene expression and enzyme synthesis related to the defense system were triggered like HSPs, and enzymes involved in the ascorbate-glutathione cycle (Ma et al., 2008). Heat

tolerant species may succeed in repairing heat stress damages while heat susceptible species failed to repair the damages. Their proteins will degrade and tissues will be damaged. In some plant, proteins related to the ascorbate-glutathione cycle increase at the beginning of heat stress. The activity of those proteins eventually decreased. The changes in amount are different between varieties.

2.1.2 Reactive oxygen species and heat stress

One effect of heat stress is the stimulation of ROS accumulation. In plants, both free radical forms such as the super oxide anions ($O_2^{\cdot -}$), the hydroxyl radicals ($OH^{\cdot}$) and $OH_2^{\cdot}$ and non-radical forms such as the H_2O_2 of ROS were observed (Edreva, 2005; Iba, 2002). They are the by-products from the process of the consumption of O_2 during photosynthesis. Chlorophyll and mitochondria, where the main oxidative-reduction reactions occur, are the major sources of ROS. ROS are also produced in plasma membrane, nuclei and peroxisomes (Kristiansen et al., 2009). The leakage of electrons from electron-transport chains and excessive absorbed light energy also cause the synthesis of ROS (Edreva, 2005).

ROS has dual-roles in plant physiology processes, toxic compound and signal molecule (Edreva, 2005)**.** High content of ROS is toxic to the enzymes and plant tissues. Low levels of ROS function as signals which trigger the defense system under various biotic and abiotic stresses.

Heat stress can cause the increase of ROS content (Ma et al., 2008; Suzuki and Mittler, 2006). The antioxidants and antioxidative enzymes related to the scavenging of

ROS also change due to high temperature stress (Ma et al., 2008). Heat-sensitive varieties usually have higher ROS content than heat-tolerant varieties under heat stress.

Plants have developed several pathways to scavenge excessive ROS. The major pathways are the ascorbate-glutathione cycle, the thioredoxin system, non-enzymatic $OH_2^{\cdot}$ scavenging, and thermal dissipation via the xanthophylls cycle (Edreva, 2005; Smith and Cheng, 2005). Ascorbate-glutathione is an enzymatic process and five enzymes are involved in this process (Fig 2.1) (Smith and Cheng, 2005). In this process $O_2^{\cdot -}$ and H_2O_2 were transformed to malondialdehyde (MDA) and then detoxified by enzymes to non-radio form. In the chloroplast, enzyme groups are the target of H_2O_2 and the damage will inhibit CO_2 fixation. Ascorbate peroxidase (APX) is the main enzyme to reduce the H_2O_2 level. $OH_2^{\cdot}$ is quenched by ascorbate, tocopherols and glutathione. Like $OH_2^{\cdot}$, $^1O_2$ is also not scavenged by enzymes. This toxic compound is broken down by the xanthophylls cycle. In the xanthophylls cycle, excessive light and energy are dissipated to heat through the conversion of violaxanthin to the antheraxanthin and then to the zeaxanthin. The efficiency of plants capability to scavenge excessive ROS is crucial to plant heat tolerance.

2.1.3 High irradiance and heat stress

Light is the most important environmental factor for plants. Plants absorb the sun light and transform it into the energy needed for metabolism. However, excessive light is deleterious to plants and causes photoinhibition, especially when combined with other environmental stresses such as heat stress. Kislyuk et al. (2004) found that the ability to

fix CO_2 in wheat leaves was reduced twice as much under heat stress in the light than in the dark.

The photosynthetic apparatus is the most sensitive plant part to high irradiance and photosystem II is more susceptible than photosystem I (Barth et al., 2001; Guo et al., 2006; Khatoon et al., 2009). Damage to the photosynthetic apparatus will cause the inhibition of photosynthesis activities. Volkova et al. (2009) found that high irradiance can cause a decline in predawn quantum yield of photochemistry, maximal Rubisco activity and electron transport capacity. Their research also showed that heat stress caused a further decline in predawn quantum yield of photochemistry and caused chlorophyll bleaching. The impairment of PS II is caused by the degradation of the D1 protein and the failure of repairing PS II (Khatoon et al., 2009). High light can cause unstacking of thylakoids and is proposed to be a mechanism of plants to resist high irradiance and prevent further damage of D1 protein because photoinhibition on unstacked thylakoids was significantly lower than stacked thylakoids (Khatoon et al., 2009). High light also stimulated the elevated contents of zeaxanthin, antheraxanthin and deepoxidation state (Guo et al., 2006), the non-photochemical quenching system which protects the plants from oxidative stress. These might explain the various effects of light by irradiance dose. Kislyuk et al. (2008) found that, under moderate heating, low and moderate light increased the leaves' CO_2 uptake compared with no light and excessive light conditions. Even at higher temperatures low light still has a protection action (Kislyuk et al., 2008).

2.2 Iron nutrient and iron uptake

Iron is one of the essential nutrients for plant growth. It plays many crucial roles in cellular activity and physiological processes including chlorophyll synthesis, electron transfer in photosynthesis, enzyme function, and DNA synthesis (Gonzalez-Vallejo, 2000). Iron is a main component of the Earth's crust, however it is not soluble and cannot be directly used by plants. Iron deficiency is very common in plants. Ivy geranium is sensitive to high temperatures and the heat damage in ivy geraniums might relate to iron nutrient availability, uptake, or use in the plant (Dhir, 2008). Application of chelated iron is colloquially reported to enhance heat tolerance and reduce the foliar bleaching in ivy geranium caused by high temperatures.

2.2.1 Role of Iron in Photosynthesis and Photoprotection

Iron is important in chlorophyll biosynthesis and the plant's photoprotection. Iron is the main component of many key proteins in chloroplasts. Reduction of photosynthesis caused by iron deficiency has been reported in many plants. Iron deficiency can decrease leaf chlorophyll concentration, photosynthesis rate, RuBP carboxylation capacity, Rubisco enzyme activation and gene expression (Andaluz et al., 2006; Larbi et al., 2006). Subsequently, iron deficiency leads to reduction of plant yield. High accumulation of iron in young and old leaves may attribute to iron deficiency tolerance (Mahmoudi et al., 2005).

The effects of iron application on plants for photoprotection have been studied. The two major photoprotective mechanisms are singlet oxygen quenching and thermal dissipation via the xanthophyll cycle, and scavenging of ROS through the ascorbate-glutathione cycle (Smith and Cheng, 2005). The photoprotective mechanisms in leaves of

a low soil pH tolerant grape were enhanced with an increase in iron application (Smith and Cheng, 2005). When iron was reapplied to iron-deficient sugar beet, the amount of zeaxanthin and photosystem II efficiency increased. Non-photochemical quenching (NPQ) and thermally dissipated energy decreased before chlorophyll synthesis (Larbi et al., 2004).

The reason for iron-deficiency mediated chlorosis is not very clear. One assumption is that lack of iron inhibits chlorophyll biosynthesis by failing to provide essential materials. Another theory is that iron caused a photo-oxidative progress interaction with the development of chlorosis, because under lower levels of photosynthetic photon flux density than those used for plant growth, iron-deficient leaves could re-green (Heras, 1960; Larbi et al., 2006). Both of these can lead to chlorosis.

2.2.2 Iron Uptake and Ferric Chelate Reductase

There are two main strategies for iron acquisition in plants named strategy I and strategy II. Besides grasses, most higher plants are strategy I plants which reduce ferric iron before uptake. This process is mediated by a plasma membrane–bound redox system (Schmidt, 2003). Iron is first reduced from ferric iron to ferrous iron by ferric chelate reductase (FCR) on the root membrane. This step is required for iron acquisition. Then iron is oxidized and transported as Fe^{3+}-citrate complex for long-distance transport in the xylem from roots to shoots (Hell and Stephan, 2003). To be assimilated by the leaf, ferric iron should be reduced to the ferrous form again by FCR in the leaf or other tissues.

FCR is crucial in iron uptake. Temperature, iron amount inside and outside the plant and light affect FCR activity. Rivero (2003) reported that high temperatures (35℃)

for tomato and low temperatures (10℃) for watermelon significantly reduced FCR activity in roots and caused total and free iron to decrease in the roots and leaves. FCR activity is induced by iron deficiency (Schmidt, 2003). In another report (Gonzalez-Vallejo et al., 2000), iron deficiency decreased the FCR in the leaf. The optimal pH values for FCR activity were 5.5 and 5.5 to 6.0 in the mesophyll protoplasts isolated from Fe-deficient and Fe-sufficient plants, respectively. Their data supports that pH values of 7.0 or higher in the apoplast would cause the activity of the mesophyll cell FCR to decrease markedly (Gonzalez-Vallejo et al., 2000). In many cases, leaves with low-chlorophyll (Chl) from iron-deficient plants have total leaf iron concentrations similar to those found in iron-sufficient plants, the so-called "chlorosis paradox" (Abaddia et al., 1984; Morale et al., 1998). Leaf FCR regulation shows some differences from root FCR. The genes of FCR, named FRO, were cloned from *Arabidopsis*, tomato, pea and maize. Currently, the study of the enzyme activity can be conducted on a molecular biology level. Studies found the FRO gene is light-regulated and tissue-specific and it expresses cell differentiation-specifically (Feng et al., 2006).

2.3 Laboratory Based Variety Selection Techniques

'Selection' is the essential method for plant breeding and it has been used for thousands of years. Humans first simply selected plants with desired traits directly from nature. Then humans produced new plants by crossbreeding and selecting from the plants they propagated. Nowadays, with the new biology techniques such as tissue culture and molecular biology, it is more efficient to create plant pools with desired traits. There are

still a large number of plants in these pools. To increase the efficiency of screening, selection is still very important.

The most traditional selection method is to grow the plants in the field and then compare their performance. Up till now, this is the most accurate method to find the best varieties; however, this is also the most time and cost consuming one. In the past 40 years, scientists have been trying to establish more efficient selection techniques. In heat tolerance plant breeding area, scientists have developed several laboratory-based methods, such as cell membrane thermostability test (CMT), Tetrazolium Triphenyl Chloride Test (TTC) and in-vitro pollen test (Gajanayake et al., 2011; Yeh and Lin, 2003)**.** All these methods evaluate the high temperature tolerance of plants by measuring the physiological and morphological changes of plants under heat stress. These methods significantly increase the efficiency of breeding.

2.3.1 Cell Membrane Thermostability

Cell membrane thermostability (CMT) under heat stress is a main plant trait for heat tolerance. Increased leakage of cells under heat stress causes degradation of proteins. A rapid and efficient method was initially developed to quantify CMT by measuring the electrolyte leakage of sorghum leaf discs under heat stress (Marcum, 1998; Sullivan, 1979). This method has been widely used to measure CMT in many plants. Marcum (1998) used CMT test to evaluate heat tolerance in turfgrasses and CMT test results showed high correlation with leaf firing and shoot dry weight. Srinivasan et al. (1996) use this method explore the differences of heat tolerance of four food legumes. Research also found some correlation between CMT test results and other heat stress induced changes. Yeh and Lin (2003) found that relative injury negative related to heat-induced delay to

flowering in chrysanthemum. By far, this technique has been applied on a wide range of agronomic plants species including soybean (Martineau et al., 1979), tomato (Chen et al., 1982), wheat (Saadalla et al., 1990), and potato (Chen et al., 1982), as well as horticulture plants such as cabbage (Fokar et al., 1998), holly (Ruter, 1993), Kentucky bluegrass (Marcum, 1998) and English ivy (Yeh and Hsu, 2004). These studies suggest that CMT is a rapid and cost-saving laboratory method for screening plant varieties for heat tolerance.

2.3.2 Tetrazolium Triphenyl Chloride (TTC) Test

The Tetrazolium Triphenyl Chloride (TTC) cell viability assay is based on the principle that a decrease in cell viability is always accompanied by a decrease in tetrazolium salt reduction, which is accomplished by the cellular redox system and is an indicator of respirational activity (Dhanda and Munjal, 2006).

The TTC assay was first used for measuring seed viability (Laken, 1949). Then this method was widely adapted to use in testing the viability of plant tissues under heat stress (Duncan and Widholm, 2004). Like the CMT assay, the TTC assay has been used for evaluation of plant varieties for heat tolerance, particularly in wheat. Dhanda and Munjal (2006) used TTC assay to evaluate 28 diverse wheat genotypes which were grown under normal and heat stress conditions for two years. In their research, they also used CMT test, heat susceptibility index (HSI) and heat response index (HRI). Their research shows that TTC is one of the most important indicators of heat tolerance.

2.3.3 In-Vitro Pollen Germination and Pollen Tube Length (PG&PTL)

CMT and TTC methods can be applied on a wide range of plant species. For some fruit bearing species, measurement of in-vitro pollen germination and pollen tube growth

might provide more accurate test results than CMT and TTC tests. Reproductive organs in plants are more sensitive to heat stress than other parts of the plants (Lyakh et al., 1991; Reddy and Kakani, 2007). Pollen germination and pollen tube growth were significantly inhibited under high temperature (Zinn et al., 2010). The effects of high temperature on pollen development varies among varieties. Therefore, PG&PTL test can be useful in a heat tolerance breeding program. Currently, the application of this technique to evaluate heat stress resistance has been studied in agronomic crops such as maize and cotton (Kakani et al., 2005; Lyakh et al., 1991). This method has also been studied on ornamental plants such as ornamental peppers (Gajanayake et al., 2011). These studies indicate the PG&PTL test may be useful to evaluate the heat tolerance of other ornamental plants.

2.3.4 Conclusion of Laboratory Based Variety Selection Techniques

Though laboratory based methods are very efficient for evaluating heat tolerance in breeding programs, they cannot be used alone. CMT, TTC and PG&PTL methods are still not as accurate as field trials. The aim of using these tests is to reduce the selection pool of plants and reduce the cost and time of breeding new varieties. Newer techniques include the molecular marker technique which is currently widely used in plant breeding. Compared to the laboratory based tests, molecular marker technique is more sophisticated but usually costs more time and resources to find the proper marker. This technology still needs to use field trial results or other laboratory methods results as a control to test the results. For breeders to whom molecular biology methods are not practical, cost efficient and less sophisticated plant physiology theory based methods are more realistic.

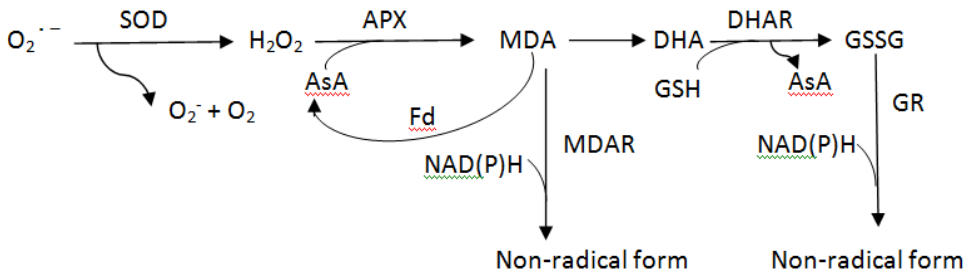

Figure 2.1 ROS enzymatic scavenging mechanism: Ascorbate-glutathione cycle

2.4 Literature Cited

Abaddia, J., E. Monge, L. Montanes and L. Heras. 1984. Extraction of iron from plant leaves by iron II chelator. Journal of Plant Nutrition 7: 777-784.

Andaluz, S., A.F. Lopez-Millan, J.D. Rivas, E.M. Aro, J. Abadia and A. Abadia. 2006. Proteomic profiles of thylakoid membranes and changes in response to iron deficiency. Photosynthesis Research 89: 141-155.

Barth, C., G.H. Krause and K. Winter. 2001. Responses of photosystem I compared with photosystem II to high-light stress in tropical shade and sun leaves. Plant Cell and Environment 24: 163-176.

Chen, H.H., Z.Y. Shen and P.H. Li. 1982. Adaptiability of crop plants to high temperature stress. Crop Sci 22: 719-725.

Dhanda, S.S. and R. Munjal. 2006. Inheritance of cellular thermotolerance in bread wheat. Plant Breeding 125: 557-564.

Dhir, R. 2008. Causes of whitening of ivy geraniums (*Pelargonium peltatum*). Ph.D. Dissertation, Mississippi State University, Starkville, MS

Duncan, D.R. and J.M. Widholm. 2004. Osmotic induced stimulation of the reduction of the viability dye 2,3,5-triphenyltetrazolium chloride by maize roots and callus cultures. Journal of Plant Physiology 161: 397-403.

Edreva, A. 2005. Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach. Agriculture Ecosystems and Environment 106: 119-133.

Farooq, M., H. Bramley, J.A. Palta and K.H.M. Siddique. 2011. Heat stress in wheat during reproductive and grain-filling phases. Critical Reviews in Plant Sciences 30: 491-507.

Feng, H.Z., F.Y. An, S.Z. Zhang, Z.D. Ji, H.Q. Ling and J.R. Zuo. 2006. Light-regulated, tissue-specific, and cell differentiation-specific expression of the *Arabidopsis* Fe(III)-chelate reductase gene AtFRO6. Plant Physiology 140: 1345-1354.

Fokar, M., H.T. Nguyen and A. Blum. 1998. Heat tolerance in spring wheat I. Estimating cellular thermotolerance and its heritability. Euphytica 104: 1-8.

Gajanayake, B., B.W. Trader, K.R. Reddy and R.L. Harkess. 2011. Screening ornamental pepper cultivars for temperature tolerance using pollen and physiological parameters. Hortscience 46: 878-884.

Gonzalez-Vallejo, E.B., F. Morales, L. Cistue, A. Abadia and J. Abadia. 2000. Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant

Physiology 122: 337-344.
Guo, Y.P., D.P. Guo, H.F. Zhou, M.J. Hu and Y.G. Shen. 2006. Photoinhibition and xanthophyll cycle activity in bayberry (*Myrica rubra*) leaves induced by high irradiance. Photosynthetica 44: 439-446.

Hell, R. and U.W. Stephan. 2003. Iron uptake, trafficking and homeostasis in plants. Planta 216: 541-551.

Heras, L. 1960. Influence of light intensity on the redox potential in leaves in cases of iron-induced chlorosis. Nature 188.

Iba, K. 2002. Acclimative response to temperature stress in higher plants: Approaches of gene engineering for temperature tolerance. Annual Review of Plant Biology 53: 225-245.

Kakani, V.G., K.R. Reddy, S. Koti, T.P. Wallace, P.V.V. Prasad, V.R. Reddy, D. Zhao. 2005. Differences in in vitro pollen germination and pollen tube growth of cotton cultivars in response to high temperature. Annals of Botany 96: 59-67.

Khatoon, M., K. Inagawa, P. Pospisil, A. Yamashita, M. Yoshioka, B. Lundin, J. Horie, N. Morita, A. Jajoo, Y. Yamamoto and Y. Yamamoto. 2009. Quality control of photosystem II thylakoid unstacking is necessary to avoid future damage to the D1 protein and to facilate D1 degradation under light stress in spinach thylakoids. Journal of Biological Chemistry 284: 25343-25352.

Kislyuk, I.M., L.S. Bubolo, O.D. Bykov, I.E. Kamentseva and O.A. Sherstneva. 2008. Protective and injuring action of visible light on photosynthetic apparatus in wheat plants during hyperthermia treatment. Russian Journal of Plant Physiology 55: 613-620.

Kislyuk, I.M., L.S. Bubolo, T.V. Paleeva and O.A. Sherstneva. 2004. Heat-induced increase in the tolerance of the wheat photosynthetic apparatus to combined action of high temperature and visible light: CO_2 fixation, photosynthetic pigments, and chloroplast ultrastructure. Russian Journal of Plant Physiology 51: 455-463.

Kristiansen, K.A., P.E. Jensen, I.M. Moller and A. Schulz. 2009. Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H(2)DCFDA and confocal laser microscopy. Physiologia Plantarum 136: 369-383.

Laken, G. 1949. The topographical tetrazolium method for determining the germination capacity of seeds. Plant Physiol 24: 389-394.

Larbi, A., A. Abadia, F. Morales and J. Abadia. 2004. Fe resupply to Fe-deficient sugar beet plants leads to rapid changes in the violaxanthin cycle and other photosynthetic characteristics without significant de novo chlorophyll synthesis. Photosynthesis Research 79: 59-69.

Larbi, A., A. Anunciiacion, A. Javier and M. Fermin. 2006. Down co-regulation of light absorption, photochemistry, and carboxylation in Fe-deficient plants growing in different environment. Photosynthesis Research 89: 113-126.

Lyakh, V.A., A.N. Kravchenko, A.I. Soroka and E.N. Dryuchina. 1991. Effects of high-temperatures on mature pollen grains in wild and cultivated maize accession. Euphytica 55: 203-207.

Ma, Y.H., F.W. Ma, J.K. Zhang, M.J. Li, Y.H. Wang and D. Liang. 2008. Effects of high temperature on activities and gene expression of enzymes involved in ascorbate-glutathione cycle in apple leaves. Plant Science 175: 761-766.

Mahmoudi, H., K. Riadh, G. Mohamed and L. Mokhtar. 2005. Differences in responses to iron deficiency between two legumes: lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*). Journal of Plant Physiology 162: 1237-1245.

Marcum, K.B. 1998. Cell membrane thermostability and whole-plant heat tolerance of Kentucky bluegrass. Crop Science 38: 1214-1218.

Morale, F., A. Abadia and J. Abadia. 1998. Photosynthesis quenching of chlorophyll fluorescenceand thermal energy dissipation in iron-deficient sugar beet leaves. Journal of Plant Physiology 25: 403-412.

Martineau, J.R., J.E. Specht, J.H. Williams and C.Y. Sullivan. 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability. Crop Science 27: 579-585.

Reddy, K.R. and V.G. Kakani. 2007. Screening *Capsicum* species of different origins for high temperature tolerance by in vitro pollen germination and pollen tube length. Scientia Horticulturae 112: 130-135.

Rivero, R.M., E. Sanchez, J.M. Ruiz and L. Romero. 2003. Influence of temperature on biomass, iron metabolism and some related bioindicators in tomato and watermelon plants. Journal of Plant Physiology 160: 1065-1071.

Ruter, J.M. 1993. Foliar heat tolerance of two hybrid hollies. Hortscience 28: 650-652.

Saadalla, M.M., J.S. Quick and J.F. Shanahan. 1990. Heat tolerance in winter wheat: II. membrane thermostability and field performance. Crop Science 30: 1248-1251.

Schmidt, W. 2003. Iron solutions: acquisition strategies and signaling pathways in plants. Trends in Plant Science 8: 188-193.

Sharkey, T.D. 2005. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant Cell and Environment 28: 269-277.

Smith, B.R. and L. Cheng. 2005. Photoprotective mechanisms of 'Concord' grape leaves in relation to iron supply. Journal of the American Society for Horticultural Science 130: 331-340.

Srinivasan, A., H. Takeda and T. Senboku. 1996. Heat tolerance in food legumes as evaluated by cell membrane thermostability and chlorophyll fluorescence techniques. Euphytica 88: 35-45.

Sullivan, C.Y. 1979. Selecting for drought and heat resistance in grain sorghum. In: M. H. and S. R., editors, Stress physiology in crop plants. Wiley & Sons, New York. p. 263-281.

Suzuki, N. and R. Mittler. 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiologia Plantarum 126: 45-51.

Todorov, D.T., E.N. Karanov, A.R. Smith and M.A. Hall. 2003. Chlorophyllase activity and chlorophyll content in wild type and eti 5 mutant of *Arabidopsis thaliana* subjected to low and high temperatures. Biologia Plantarum 46: 633-636.

Volkova, L., M. Tausz, L.T. Bennett and E. Dreyer. 2009. Interactive effects of high irradiance and moderate heat on photosynthesis, pigments, and tocopherol in the tree-fern *Dicksonia antarctica*. Functional Plant Biology 36: 1046-1056.

Wahid, A., S. Gelani, M. Ashraf and M.R. Foolad. 2007. Heat tolerance in plants: An overview. Environmental and Experimental Botany 61: 199-223.

Wassmann, R., S.V.K. Jagadish, S. Heuer, A. Ismail, E. Redona, R. Serraj, R.K. Singh, G. Howell, H. Pathak, and K. Sumfleth. 2009. Climate change afffecting rice production : the physiological and agronomic basis for possible adaptation strategies. In: D. L. Sparks, editor Advances in Agronomy, Vol 101. Elsevier Academic Press Inc, San Diego. p. 59-122.

Xu, C. and B. Huang. 2008. Root proteomic responses to heat stress in two *Agrostis* grass species contrasting in heat tolerance. Journal of Experimental Botany 59: 4183-4194.

Yeh, D.M. and P.Y. Hsu. 2004. Heat tolerance in english ivy as measured by an electrolyte leakage technique. Journal of Horticultural Science and Biotechnology 79: 298-302.

Yeh, D.M. and H.F. Lin. 2003. Thermostability of cell membranes as a measure of heat tolerance and relationship to flowering delay in chrysanthemum. Journal of the American Society for Horticultural Science 128: 656-660.

Zinn, K.E., M. Tunc-Ozdemir and J.F. Harper. 2010. Temperature stress and plant sexual reproduction: uncovering the weakest links. Journal of Experimental Botany 61: 1959-1968.

CHAPTER III

RESPONSES OF H_2O_2 CONTENT, ROS SCAVENGING ENZYMES ACTIVITIES AND ACTIVE IRON CONTENT IN IVY GERANIUM TO HEAT STRESS

3.1 Abstract

Ivy geranium (*Pelargonium peltatum*) is a heat susceptible species with its heat tolerance varying among varieties. Reactive oxygen species (ROS) and in-vivo defense systems are related to plant heat damage and heat tolerance. Application of chelated-iron was reported to enhance ivy geranium heat tolerance; however, the correlation of ROS, relative enzyme stability, and iron content to differences in heat tolerance in ivy geraniums is unknown. Here we show that the H_2O_2 content and ROS scavenging enzyme stability in ivy geranium varies with varieties and active iron is not related to heat tolerance in ivy geranium. We found that H_2O_2 content in mature leaves in both heat tolerant 'Beach' and sensitive 'Butterfly' increased under heat stress but 'Butterfly' had a relatively greater increase of this toxic compound. Catalase (CAT) activities in young leaves in both varieties decreased. In young leaves of 'Butterfly', CAT activities decreased to a level significantly lower than that in old leaves while this did not occur in 'Beach'. Superoxide dismutase (SOD) activities in 'Butterfly' young leaves were also decreased. All these phenomenon coincided with the heat tolerance differences of the two varieties. However, active iron content only changed with leaf age and did not vary between varieties or treatments. Our results demonstrated that ROS scavenging ability

and relative enzyme stability may be the cause of the variance in heat tolerance in the different ivy geranium varieties and that iron deficiency was not the cause of heat damage.

Key Words: Heat stress, Reactive oxygen species, CAT, SOD, Chelated-iron

3.2 Introduction

Ivy geraniums are an important bedding plant species for the U.S. floriculture industry (NASS, 2012). Though very tolerant to low temperature and drought, ivy geraniums do not always perform well in hot summers especially in the southern U.S. ROS are always associated with heat stress tolerance and heat damage in plants (Suzuki and Mittler, 2006). Small amounts of ROS synthesized in-vivo under heat stress works as a signal to stimulate the intro resistance system to reduce the damage caused by heat stress. Excessive synthesis of ROS causes damage to important enzymes and organs in plants. Iron is also reported to be associated with ivy geranium heat tolerance (Dhir, 2008); however, ROS content and the relative enzyme and iron content have not been studied.

Heat stress adversely affects plant growth and development by causing morpho-anatomical, physiological, biochemical changes in plants (Wahid et al., 2007). Photosynthesis, the most important physiological process in plants, is considered the most sensitive and susceptible (Allakhverdiev et al., 2008). ROS plays a crucial role in the damage and recovery of the photosynthesis system under heat stress. ROS, in the form of $O_2^{\cdot -}$, $OH^{\cdot}$, $OH_2^{\cdot}$ and H_2O_2, are toxic chemicals which are synthesized in plants under abiotic stress (Edreva, 2005). In the photosynthesis system, ROS generation in both

the light reaction and Calvin cycle are observed. ROS can cause oxidative damage to proteins, membranes and other cell organs. A small amount of ROS works as a signal triggering the defense system of the whole plant. Plants have developed enzymatic and non-enzymatic pathways to scavenge extra ROS. The ascorbate-glutathione cycle, enzymatic pathway, is one of the most important ones. H_2O_2 is scavenged by this cycle and detoxified to H_2O and O_2. Catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and several other enzymes are involved in this process (Asada, 1999; Noctor and Foyer, 1998; Smith and Cheng, 2005). However, no research has been conducted to investigate the relationship of ROS and relative enzymes to heat tolerance in ivy geraniums.

Iron is an essential nutrient for plant growth. It plays many crucial roles in cellular activity including chlorophyll synthesis, electron transfer in photosynthesis, enzyme function, and DNA synthesis. There are two main strategies for iron acquisition in plants, strategy I and strategy II (Smith and Cheng, 2005). Besides grasses, most higher plants are strategy I plants reducing ferric iron before uptake, a process that is mediated by a plasma membrane–bound redox system (Schmidt, 2003). Iron is first reduced from ferric iron to ferrous iron by ferric chelate reductase (FCR) on the root membrane. This step is required for iron acquisition. Iron is then oxidized and transported as Fe^{3+}-citrate complex for long-distance transport in the xylem from roots to shoots (Hell and Stephan, 2003). To be assimilated by the leaf, ferric iron should be reduced to the ferrous form again by FCR in the leaf or other tissues.

Chlorotic leaves under Fe deficiency sometimes have elevated concentrations of total Fe in a phenomenon called the “chlorosis paradox”. In plants, Fe with Fe (II)-

chelators or dilute acids, which are called “active iron”, is often a better measure of Fe status than total Fe. Chlorotic leaves usually have a high total iron content but a low level of active iron which has the most function in plant physiological processes. Many growers suggested that an application of iron before heat stress starts may mitigate leaf bleaching in ivy geraniums under heat stress. However, the role of iron in ivy geranium heat tolerance is still largely unclear.

The purpose of this study is to determine the change in active iron content and H_2O_2 content in leaves of different age under heat stress. The change of CAT and SOD activities were also studied to identity whether their activity led to the changes in H_2O_2 and were affected by heat stress. This study compared two ivy geranium varieties which differ in heat tolerance, to determine the relationship of the two parameters in ivy geranium heat tolerance and the roles of CAT and SOD in ivy geranium heat tolerance. The results may provide more physiological information to explain the mechanism of heat tolerance between different ivy geranium cultivars and be useful in breeding ivy geraniums for heat tolerance.

3.3 Material and Methods

3.3.1 Plant culture

Two commercial ivy geranium varieties, heat sensitive ‘Butterfly’ and heat tolerant ‘Beach’ (Syngenta Flowers, Inc., Boulder, CO), were used in this research. Rooted cuttings were potted in 15 cm (1 L) pots in Sunshine Mix 1 (Sun Gro Horticulture, Inc., Bellevue, WA) and fertilized at every irrigation with 20-4.4-16.6 (Peter’s peat-lite 20-10-20; Scotts Co., Marysville, OH) at 250 mg $N \cdot L^{-1}$. The plants

were grown in the greenhouse at 21/18°C (d/n) for 6 weeks. The plants were then transferred to growth chambers and grown for 3 weeks at the treatment temperatures.

3.3.2 Experiment design and Treatment

The experiment was set up as a split plot design with subsamples and replicated in time. There were 3 replications with 3 subsamples and 2 varieties. Plants were transferred to growth chambers at a temperature of 35/30ºC (d/n) or 25/15ºC (d/n). The plants of the two varieties were randomized in the growth chambers. One week after the start of the treatment of the first replication, the second replication was placed into the chambers. Another week later, the third replication was placed under treatment. All replications were treated for 3 weeks. The youngest fully expanded leaf and third oldest leaf from the plant base were collected from each plant for analysis. The final results' variance was analyzed by ANOVA using Proc-GLM (SAS software, SAS Institute, Cary, NC). The statistic test results with a p value larger than 0.05 is considered significant.

3.3.3 H_2O_2 Assay

The content of H_2O_2 was determined using the method described by Macnevin (1953) and Brennan (1977) with modification. H_2O_2 and Ti^{4+} can form a specific complex which has a peak absorbance at 415 nm. 0.1 g fresh leaf sample was homogenized in 1.5 ml 50 mM K-phosphate buffer (pH 6.5). The extract was centrifuged for 15 min at 12,000 g. All of the supernatant was collected and the volume of the supernatant recorded for calculation. 0.5 ml supernatant was added to 1.5 ml test buffer (20% H_2SO_4, 0.1% $TiCl_4$) and mixed evenly. The obtained solution was centrifuged for 5 min at 13,000 g. The absorbance of the supernatant was read at 415 nm against a distilled water blank using a

spectrophotometer (Thermo Scientific Evolution 100, Thermo Fisher Scientific, Inc., Waltham, MA). The concentration of H_2O_2 in the extract was determined using the standard curve method. The content of H_2O_2 in the sample was calculated using the formula:

$$H_2O_2 \text{ content in sample } (\mu mol/mg) = \frac{C * V}{FW} \quad (3.1)$$

C = concentration of H_2O_2 in the extract (μM)

V = total volume of supernatant obtained in the first centrifugation (**L**)

FW = weight of sample (mg)

3.3.4 Active Iron Assay

The active iron content was assayed as described by Macnevin (1953) and Chen (2004) with modification. The active iron used by plants is always in the form of ferrous iron which can form a specific complex with 2, 2'-dipyridyl-HCl and be measured by colorimetry. Three 1 cm^2 discs were punched from each leaf and each disc was cut into two. The leaf discs were put into 2ml Eppendorf microcentrifuge tubes which contained 1.2 ml extraction buffer (80 mM 2, 2'-dipyridyl-HCl pH 3.0, 10% methanol). The tube was shaken for 24 h at room temperature (20°C). The extraction was filtered using 0.45-μm syringe filter. The filtered solution was assayed at 522 nm. The content of active iron was calculated using the standard curve method.

3.3.5 Tissue extraction preparation and CAT Assay

The tissue samples used for enzyme assay were frozen in liquid nitrogen and stored at -80 ºC. The tissue extraction procedure adopted was as described by Smith

(2005) with modification. 0.1 g fresh tissue was homogenized in liquid nitrogen with a pre-cooled mortar and pestle. The homogenized tissue was then moved rapidly to 2 ml pre-cooled microcentrifuge tubes which contained 1.9 ml extraction buffer (50 mM KH_2PO_4-KOH pH 7.6, 0.1 mM EDTA, 4% PVPP) and shaken for 1 min. After shaking, the extraction was centrifuged at 13,000 g for 15 min. The supernatant was transferred to another clean microcentrifuge tube and held for enzyme assay.

CAT activity was assayed by measuring the scavenging H_2O_2 in the plant extract. 120 µl of the plant extract was added to 1814 µl reaction buffer (100 mM K-phosphate; pH 7.0). Then, 66 µl H_2O_2 was added to the solution to start the reaction. The absorbance decline rate of the sample was read in the spectrophotometer at 240 nm for 1 min with a time lag of 15 sec. The CAT activity was calculated as:

$$\text{CAT activity / unit of protein } (\mu\text{mol min}^{-1}) = \Delta A * V / [K * L]/m \qquad (3.2)$$

ΔA = absorbance change per minute

V = volume of reaction system: 2ml

K = CAT extinction coefficient: 0.0394 $\mu mol^{-1}cm^{-1}$

L = width of light pathway: 1cm

m = total protein content (mg)

3.3.6 SOD Assay.

Tissue extraction procedure used for SOD assay was similar to that of the CAT assay. The only difference was the extraction buffer also contained 4% polyvinylpyrrolidone (PVP). The SOD activity is assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Shiyab, et al., 2009)**.** The total

reaction system contained 50 mM K-phosphate buffer, 13 mM Met, 0.1 mM EDTA, and 2 μM Riboflavin. Then, 20 μL tissue sample was added to the tube and mixed evenly. NBT was added to the reaction solution and the whole system volume increased to 4 ml. Three tubes without tissue samples were placed in the dark as a control and were measured when the experiments ended. The average of the results was used as the maximum absorption. All the samples were place under light at room temperature (20°C) for 30 min. After the reaction, all the samples were put into the dark to stop the reaction. The solutions were assayed at 560 nm. The SOD activities were calculated as:

$$\text{SOD Activity / unit of protein } (\mu\text{mol min}^{-1}) = (A_{max}\text{-}A) / A_{max}*0.5/m \qquad (3.3)$$

A_{max} = Maximum absorption

A = Absorption of each sample

m = total protein content (mg)

3.4 Results and Discussion

3.4.1 H_2O_2 content and CAT and SOD activities during the heat treatment

The H_2O_2 content was measured before and after the heat treatment. Under the 25ºC treatment, 'Beach' had a greater level of H_2O_2 than 'Butterfly' in both young and old leaves. Young leaves of both varieties had a greater levels of H_2O_2 than old leaves. Under the heat stress, the H_2O_2 level in young leaves of both varieties decreased; however, the H_2O_2 level in old leaves increased. Under the heat treatment, 'Beach' still had greater H_2O_2 levels than 'Butterfly'. However, the proportion of H_2O_2 level increase in 'Beach' was much smaller than that in 'Butterfly'. (Table 3.1) H_2O_2 is a member of the ROS family. It can inactivate the Calvin cycle enzymes, cross-link proteins D1 and

D2, oxidize metal-containing enzymes and cause damage to M_n cluster in PS II (Edreva, 2005; Niyogi, 1999). H_2O_2 also works as signal factors so it is normally at a balanced level in plants (Suzuki and Mittler, 2006). The level of H_2O_2 always increases under heat stress (Yin et al., 2008) and heat tolerant varieties usually maintain a lower level of H_2O_2 than heat susceptible ones (Sairam et al., 2000). The increases of H_2O_2 in old leaves in both varieties indicate both varieties were suffering heat stress and oxidative damage. As the content of H_2O_2 increase in 'Beach' was smaller, 'Beach' could more effectively scavenge ROS and was more heat tolerant than 'Butterfly'. The ability to scavenge ROS may be a main factor for determining the heat tolerance of ivy geranium varieties.

CAT and SOD activities were assayed in this research and under the control treatment (25°C), CAT activity showed no difference between young and old leaves (Table 3.2). It was also not different between cultivars. However, under heat stress, CAT activity in both cultivars decreased in young leaves compared to the control while in old leaves the decrease was not significant. The two cultivars showed some differences in the extent of the CAT activity decrease when under heat stress. Though CAT activity in 'Butterfly' young leaves was not significantly lower than that in 'Beach' young leaves, it was significantly lower than that in 'Butterfly' old leaves when under heat stress. The CAT activity levels in 'Beach' under heat stress did not significantly differ between young and old leaves. The main function of CAT is to decompose H_2O_2 to O_2. Heat stress may also cause CAT degradation. Anderson (2002) found that CAT activity in pepper leaves significantly decreased when heat damage occurred. He also found, compared to young leaves, old leaves are more stable and resistant to heat stress. This is identical to the results found in this research. As heat stress only caused a significant decrease in

CAT activity in young leaves of 'Beach' compared to old leaves in 'Butterfly', 'Butterfly' suffered more serious heat damage compared to 'Beach' confirming 'Butterfly' is less heat tolerant than 'Beach'. Even in old leaves, 'Butterfly' showed a slight trend towards a decrease in CAT activity though the data were not significant. This may be found to be significant if both cultivars were exposed to a longer heat stress treatment. The results showed that CAT enzymes may be one of the targets of heat stress damage. The loss of CAT function might be one of the causes of H_2O_2 level increase in the old leaves of both cultivars under heat stress. As the loss of CAT function is not highly significant, there must be other ROS scavenging enzymes involved in this process causing the differences between the two cultivars.

The changes of SOD activities are slightly different from CAT. There is no difference in SOD activity between the two varieties (Table 3.3). In old leaves, heat stress did not cause a decrease of SOD. In young leaves, though there was no difference between varieties, SOD activity decreased significantly under heat stress in 'Butterfly' but not in 'Beach'. SOD catalyzes the O_2^- into H_2O_2 and O_2 (Alscher et al., 2002) and is very important for scavenging ROS. The results showed that SOD in 'Beach' was more stable than in 'Butterfly'. Under heat stress, the levels of both CAT and SOD activity were decreased and these key enzymes help plants resist heat stress.

3.4.2 Active Iron content during the heat treatment

Active iron content in both varieties were not significantly different in regards to leaf age or temperature treatment. For each variety, iron content in young leaves was lower than in old leaves and this content did not change under the heat treatment. (Table 3.4). According to industry experience, application of chelated-iron before heat stress can

increase heat tolerance. Young leaves which have lower iron content are less heat tolerant than old leaves. A previous study found heat stress caused changes in iron content in tomato and watermelon (Rivero et al., 2003). Iron usually accumulates in old leaves and affects chlorophyll content. So, there is the possibility that total iron content changes under heat stress. This change, which may be due to iron immobilization or an absorption problem, could be the cause of heat damage in ivy geraniums. However, in our research, no iron deficiency was found in either variety under heat stress.

3.5 Conclusions

H_2O_2 increases under heat stress with heat tolerant varieties typically maintaining a lower level than heat susceptible varieties. Increases in H_2O_2 levels due to high temperature treatments in old and young leaves of both varieties indicated both 'Beach' and 'Butterfly' suffered heat stress and oxidative damage. The H_2O_2 increase in old leaves of 'Beach' was less than in 'Butterfly' indicating 'Beach' may be more effective at scavenging ROS and thus more heat tolerant than 'Butterfly'.

CAT and SOD activity was more stable in the heat tolerant variety 'Beach' than in the heat sensitive variety 'Butterfly'. Heat stress decreased the activity levels of both CAT and SOD, key enzymes helping plants resist heat stress. The mechanism heat tolerant varieties use to keep the levels of these enzymes stable needs further investigation.

Active iron content did not vary between leaf age or temperature treatment. However, chelated iron applications may improve heat tolerance in ivy geraniums by supplying sufficient iron to stimulate heat protection system enzyme synthesis.

Table 3.1 Change in H_2O_2 (µmol/mg fresh tissue) content in young and old leaves of ivy geranium 'Beach' (heat tolerant) and 'Butterfly' (heat sensitive) grown under two temperature treatments, control (25/15ºC d/n) and heat stress (35/30ºC d/n).

		H_2O_2 Content (µmol/mg fresh tissue)		
		Cultivars Name		
Leaf age	Temperature Treatments	'Butterfly'	'Beach'	
Young	25/15ºC	11.09	19.86	*
	35/30ºC	2.87	5.63	*
H_2O_2 content changes between two temperature treatments (%)		-74.12% *	-71.65% *	
Old	25/15ºC	4.13	6.88	*
	35/30ºC	7.00	9.25	*
H_2O_2 content changes between two temperature treatments (%)		+69.49% *	+34.00% *	

*significant at $P<0.05$, under ANOVA test

Table 3.2 Change in catalase (CAT) (µmol·min^{-1}·mg^{-1}) activity in young and old leaves of ivy geranium 'Beach' (heat tolerant) and 'Butterfly' (heat sensitive) grown under two temperature treatments, control (25/15ºC d/n) and heat stress (35/30ºC d/n).

		CAT (µmol·min^{-1}·mg^{-1})		
		Cultivars Name		
Leaf age	Temperature	'Butterfly'	'Beach'	
Young	25 /15ºC	0.39	0.37	NS
	35/30ºC	0.10 *	0.17 *	NS
Old	25/15ºC	0.29	0.30	NS
	35/30ºC	0.27 NS	0.30 NS	NS

*=significant, NS=not significant at $P<0.05$, under ANOVA test
LSD within row and column.

Table 3.3 Change in superoxide dismutase (SOD) (μmol·min^{-1}·mg^{-1}) activity in young and old leaves of ivy geranium 'Beach' (heat tolerant) and 'Butterfly' (heat sensitive) grown under two temperature treatments, control (25/15ºC d/n) and heat stress (35/30ºC d/n).

		SOD (μmol·min^{-1}·mg^{-1})		
Leaf age	Temperature	Butterfly	Beach	
Young	25/15ºC	1.54	1.57	NS
	35/30ºC	0.70	1.17	NS
		*	NS	
Old	25/15ºC	1.34	1.44	NS
	35/30ºC	1.71	1.24	NS
		NS	NS	

*=significant, NS=not significant at $P<0.05$, under ANOVA test

Table 3.4 Leaf active iron content (mg·m^{-2}) in young and old leaves of ivy geranium 'Beach' (heat tolerant) and 'Butterfly' (heat sensitive) grown under two temperature treatments, control (25/15ºC d/n) and heat stress (35/30ºC d/n).

		Active iron content (mg·m^{-2})		
Leaf age	Temperature	Butterfly	Beach	
Young	25/15ºC	7.15	7.84	NS
	35/30ºC	7.92	6.70	NS
		NS	NS	
Old	25/15ºC	11.39	10.97	NS
	35/30ºC	10.60	11.09	NS
		NS	NS	

*=significant, NS=not significant $P<0.05$, under ANOVA test

3.6 Literature Cited

Allakhverdiev, S.I., V.D. Kreslavski, V.V. Klimov, D.A. Los, R. Carpentier and P. Mohanty. 2008. Heat stress: An overview of molecular responses in photosynthesis. Photosynth Res 98: 541-550.

Alscher, R.G., N. Erturk and L.S. Heath. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 53: 1331-1341.

Anderson, J.A. 2002. Catalase activity, hydrogen peroxide content and thermotolerance of pepper leaves. Sci Hortic 95: 277-284.

Asada, K. 1999. The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Phys 50: 601-639.

Brennan, T. and C. Frenkel. 1977. Involvement of hydrogen-peroxide in regulation of senescence in pear. Plant Physiol. 59: 411-416.

Chen, L.S., B.R. Smith and L.L. Cheng. 2004. CO_2 assimilation, photosynthetic enzymes, and carbohydrates of 'Concord' grape leaves in response to iron supply. J. Am. Soc. Hortic. Sci. 129: 738-744.

Dhir, R. 2008. Causes of whitening of ivy geraniums (*Pelargonium peltatum*). Ph.D. Dissertation, Mississippi State University, Starkville, MS

Edreva, A. 2005. Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach. Agr. Ecosyst. Environ. 106: 119-133.

Hell, R. and U.W. Stephan. 2003. Iron uptake, trafficking and homeostasis in plants. Planta 216: 541-551.

Macnevin, W.M. and P.F. Urone. 1953. Separation of hydrogen peroxide from organic hydroperoxides - application to polarographic analysis of mixtures. Anal. Chem. 25: 1760-1761.

National Agricultural Statistics Service (NASS). 2012. United States Department of Agriculture. March 28, 2014. <http://usda01.library.cornell.edu/usda/current/FlorCrop/FlorCrop-04-25-2013.pdf>.

Niyogi, K.K. 1999. Photoprotection revisited: Genetic and molecular approaches. Annu. Rev. Plant Phys. 50: 333-359.

Noctor, G. and C.H. Foyer. 1998. Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Phys. 49: 249-279.

Rivero, R.M., E. Sanchez, J.M. Ruiz and L. Romero. 2003. Influence of temperature on biomass, iron metabolism and some related bioindicators in tomato and watermelon plants. J. Plant Physiol. 160: 1065-1071.

Sairam, R.K., G.C. Srivastava and D.C. Saxena. 2000. Increased antioxidant activity under elevated temperatures: A mechanism of heat stress tolerance in wheat genotypes. Biol. Plantarum 43: 245-251.

Schmidt, W. 2003. Iron solutions: acquisition strategies and signaling pathways in plants. Trends Plant Sci. 8: 188-193.

Shiyab, S., J. Chen, F.X.X. Han, D.L. Monts, F.B. Matta, M.M. Gu, Y. Su, Ma. Masad. 2009. Mercury-induced oxidative stress in Indian mustard (*Brassica juncea* L.). Environ. Toxicol. 24: 462-471.

Smith, B.R. and L.L. Cheng. 2005. Photoprotective mechanisms of 'Concord' grape leaves in relation to iron supply. J. Am. Soc. Hortic. Sci. 130: 331-340.

Suzuki, N. and R. Mittler. 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiol. Plantarum 126: 45-51.

Wahid, A., S. Gelani, M. Ashraf and M.R. Foolad. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61: 199-223.

Yin, H., Q.M. Chen and M.F. Yi. 2008. Effects of short-term heat stress on oxidative damage and responses of antioxidant system in *Lilium longiflorum*. Plant Growth Regul. 54: 45-54.

CHAPTER IV

DEVELOPING CELL MEMBRANE THERMOSTABILITY AND TRIPHYENYL TETRAZOLIUM CHLORIDE CELL VIABILITY TESTS FOR ASSESSING "HEAT TOLERANCE IN IVY GERANIUMS

4.1 Abstract

Summer heat stress causes a reduction in plant growth and flowering in ivy geraniums (*Pelargonium peltatum* L.). Current cultivar screening for heat-tolerance relies heavily on whole-plant methods which are time and labor consuming. Cell Membrane Themostability (CMT) and Triphenyl Tetrazolium Chloride (TTC) cell viability tests are alternative, laboratory-based screening methods. They have been used to screen for heat tolerance in wheat, tomato and soybean. Compared to whole-plant methods, CMT and TTC appear more efficient and cost effective; however, there is no report on application of CMT and TTC to ivy geraniums. Here we show that both CMT and TTC tests can represent the variance in heat tolerance observed in ivy geraniums. The results of both CMT and TTC tests correlated well with plant width and growth indexes. Their correlations to plant chlorosis were low. Unlike TTC, CMT strongly correlated with plant width. Our results demonstrate that CMT and TTC tests can be applied to cultivar screening for heat tolerance in ivy geraniums providing two efficient complementary laboratory-based methods to the currently used whole-plant methods for ivy geranium breeding.

Key words: cultivar screening, laboratory method, heat tolerance, cell viability

4.2 Introduction

Ivy geraniums are an important crop for the U.S. floricultural industry; however, heat stress that induces morphological and physiological changes in ivy geraniums is a main limiting factor in plant performance in the summer (Dhir et al., 2011; 2013). Evaluation and selection of varieties for heat stress rely heavily on whole-plant techniques including multi-location, multi-season field trials which provide comprehensive results (Gu and Zhang, 2012). However, these techniques are often expensive in terms of space, time, and labor. Laboratory-based methods can increase the accuracy and efficiency in breeding for heat stress (Blum et al., 2001; Dhanda and Munjal, 2006). Changes in physiological process such as cell membrane thermostability, chlorophyll content, photosynthetic performance, cell viability, pollen viability, and carbon isotope discrimination due to heat stress were reported to have potential for developing laboratory-based screening tools for heat tolerance in plants (Jiang and Huang, 2000; Lu et al., 1996; Reynolds et al., 1997; Saadalla et al., 1990; Singh et al., 2007)**.** However, the correlation between these traits and plant heat tolerance varies among different plant species and experimental conditions. CMT and TTC cell viability tests are the two most widely studied techniques and have been reported to be strongly associated with heat tolerance (Blum et al., 2001; Marcum, 1998; Singh et al., 2007).

Cell membrane thermostability under heat stress is a main plant trait for heat tolerance. Increased leakage of cell membranes under heat stress causes degradation of proteins. A rapid and efficient method was initially developed to quantify CMT by measuring the electrolyte leakage of sorghum leaf discs under heat stress (Marcum, 1998;

Sullivan, 1979). This method has been widely used to measure CMT in many plants, including soybean (Martineau et al., 1979), tomato (Chen et al., 1982), wheat (Saadalla et al., 1990), and potato (Chen et al., 1982), as well as horticultural plants such as cabbage (Fokar et al., 1998)**,** holly (Ruter, 1993), Kentucky bluegrass (Marcum, 1998), chrysanthemum (Wang and Yeh, 2008), and English ivy (Yeh and Hsu, 2004). These studies suggest that CMT is a rapid and cost-saving laboratory method for screening plant varieties for heat tolerance.

The TTC cell viability assay is based on the principle that a decrease in cell viability is always accompanied by a decrease in tetrazolium salt reduction which is accomplished by the cellular redox system and is an indicator of respirational activity (Dhanda and Munjal, 2006). The TTC assay was first used for measuring seed viability (Laken, 1949) and then widely used in testing the viability of plant tissues (Duncan and Widholm, 2004). Like the CMT assay, the TTC assay has been used for screening plant varieties for heat tolerance, particularly in wheat.

As global temperatures have risen significantly over the past decades, breeding for heat tolerance has become more important than ever. Effective and efficient cultivar screening techniques are required to reduce the breeding sample pools and shorten the breeding cycles. Laboratory-based methods provide more options for these purposes; however, there are no reports on either laboratory technique for screening for heat tolerance in ivy geraniums. The objectives of this research were to determine the extent CMT and TTC assays can be used for selecting heat-tolerance in ivy geranium cultivars and to compare the two assays for estimating variability in ivy geranium cultivars for heat

tolerance. The findings of this research are expected to provide suitable laboratory-based methods for screening ivy geraniums for heat tolerance during breeding.

4.3 Materials and Methods

4.3.1 Plant materials

Eleven ivy geranium cultivars (Syngenta Flowers, Inc., Boulder, CO) (Table 4.1) were used for the CMT and TTC assay. Rooted cuttings were potted in 15 cm (1 L) containers in Sunshine Mix 1 (Sun Gro Horticulture, Inc., Bellevue, WA) and fertilized at every irrigation with 20N-4.4P-16.6K (Peter's peat-lite 20-10-20; Scotts Co., Marysville, OH) at 250 mg $N \cdot L^{-1}$. The plants were grown in the greenhouse at 21/18°C (d/n) for 6 weeks. The plants were transferred to a growth chamber at 35°C for 24 h before being tested.

For field trials, cuttings were grown in 10-cm pots for four weeks in the greenhouse at 21/18°C (d/n) in early spring before being transplanted to 25-cm pots filled with Sunshine Mix 1 with three plants per pot. The containers were watered using drip irrigation for 20 minutes every other day throughout the summer. Each container of plants received 12g 15N-4.73P-10.79K controlled release fertilizer (Osmocote Plus 15-10-12; The Scotts Co., Marysville, OH) when first planted. Plants were grown outside in a full sun location on a gravel nursery production yard at Mississippi State University from 15 May to 1 August 2009 (average temperatures were 31/19°C, d/n).

4.3.2 CMT assay

CMT assay was conducted according to Fokar et al. (1998) with modifications. Each sample consisted of four 5-mm diameter leaf discs from the third pair of leaves

from the stem apex. The leaf discs were rinsed with distilled water three times and placed into 50 ml tubes containing 20 ml distilled water. The treatment tubes were placed in a 50°C water bath for 1 h while the control remained at 20°C. Subsequently, all samples were cooled at 10°C for 12 h and the initial solution electrical conductivity (EC) was measured using an EC meter (YSI model 35; Yellow Springs Instruments, Yellow Springs, OH). Tubes were then autoclaved for 20 minutes and the final solution EC was measured. The relative injury (RI) of the cell membranes was calculated as described by Wang et al. (2008): RI (%) =1- [1-(T_i/T_f)]/ [1-(C_i/C_f)], where T and C referred to treatment and control, respectively, and i and f referred to initial and final EC readings, respectively.

4.3.3 TTC cell viability assay

The assays were carried out as described by Porter et al. (1994) and Fokar et al. (1998) with modifications. Eight 2.0 by 0.5 cm leaf segments were excised from the third pair of leaves from the shoot apex of each plant. These segments were separated into two groups and placed separately into two 10 ml test tubes containing 100 µl distilled water. Treatment tubes were placed in a water bath for 1 h at 50°C while control tubes were kept in the refrigerator at 10°C. After incubation, 4 ml 0.8% TTC (w/v) solution was placed in each tube. All the tubes were placed under vacuum for 30 min to infiltrate TTC into the leaves. After infiltration, the tubes were placed in the dark for 12 h at 25°C and then after complete rinsing of the leaf segments with distilled water three times, 4 ml of 95% ethanol was added. Tubes were again placed in darkness for 24 h at 25°C. Afterwards, the amount of formazan dye was measured by spectrophotometer (Thermo Scientific Evolution 100; Thermo Fisher Scientific Inc., Waltham, MA) at 530 nm. Cell viability

was calculated using the formula: $V = A_t/A_c \times 100\%$ where V = Viability; At = Treatment absorbance; and Ac = Control absorbance.

4.3.4 Field trial

At the end of field trial, the two widths (W_1, W_2) and height (H) were measured. The growth index (GI) and average of width (AVEW) were calculated using the formula: $GI\ (cm^3) = 3.147 * (AVEW/2)^2 \times H$ where $AVEW\ (cm) = (W_1+W_2)/2$; W_1 = plant width (cm); and W_2 = plant width 90° to W_1 (cm). In addition, the plants were visually rated for chlorosis or leaf bleaching. A rating of 5 = no chlorosis, 4 = < 25% of the plant chlorotic, 3 = 50% of the plant chlorotic, 2 = 75% of the plant chlorotic, and 1 = 100% of the plant chlorotic.

4.3.5 Experimental design and statistical analysis

In the CMT and TTC assays and the field trial, a randomized complete design with four replications was used. Analyses of variance (ANOVA) of RI and cell viability data were analyzed using the PROC GLM procedure of SAS 9.3 (SAS Institute, Cary, N.C.). Means were separated using Duncan's Multiple Range test at 95% confidence level. Correlation analysis was also carried out to determine the relationship among RI, cell viability, and growth parameters. Levels of significance are represented by Pearson correlation coefficiency at $P = 0.05$.

4.4 Results

4.4.1 CMT Test

The RI of the 11 cultivars ranged from 33.7% to 68.2% (Fig. 4.1). The higher the RI the lower the cell membrane thermostability and the less heat tolerant the cultivar.

Among the 11 cultivars, ‘Caliente Deep Red’ and ‘Caliente Coral’ had the highest RI at above 60% and were rated to be less heat tolerant. ‘Acapulco Compact Cascade’, ‘Beach’ and ‘Blizzard Red 09’ had the lowest RI and were rated to be more heat tolerant.

4.4.2 TTC Test

In the TTC test, the cultivars which were more heat tolerant had a higher cell viability (CV) while heat susceptible cultivars had a lower CV (Fig. 4.1). The CV of the 11 cultivars ranged from 38.68% to 77.25%. In the TTC test, ‘Shiva’, ‘Blizzard Red 09’ and ‘Acapulco Compact Cascade’ were rated more heat tolerant and ‘Freestyle Artic Red’ and ‘Caliente Deep Red’ were rated less heat tolerant. The results of the TTC test were similar to CMT test results.

4.4.3 Field trial

In the field trial, plant growth index, width and chlorosis were measured (Table 4.2). In this study, growth index and plant width were used to determine the growth performance of ivy geraniums. ‘Acapulco Compact Cascade’, ‘Beach’ and ‘Blizzard Subcompact Burgundy’ had the greatest width while ‘Lambada 09’ and ‘Caliente Rose’ had the least width. ‘Acapulco Compact Cascade’ and ‘Blizzard Subcompact Burgundy’ had the greatest GI while ‘Lambada 09’ and ‘Freestyle Artic Red’ had the least GI. ‘Lambada 09’, whose growth was the least of the cultivars, had among the lowest scores for chlorosis.

4.4.4 Correlations among CMT, TTC tests and Field trial

Correlations among the three experiments were calculated (Table 4.3). CMT and TTC tests had a negative correlation of $r = 0.52$ ($P < 0.01$). The results of both tests were

similar in evaluating the heat tolerance of the 11 ivy geranium cultivars. The correlations between the laboratory tests and GI and plant width were significant. The laboratory tests had greater correlation with plant width than GI. CMT showed greater correlation with field trial results than TTC tests (Table 4.3). Chlorosis ratings did not correlate with either CMT test or TTC test.

4.5 Discussion

Cell membrane thermostability and cell respiration activity are both expected to relate to heat tolerance in plants. The results in this research support the conclusion that heat tolerance in ivy geraniums can be described in terms of CMT and TTC. This conclusion is also in agreement with previous findings in wheat and turfgrass (Dhanda and Munjal, 2006; Duncan and Widholm, 2004; Fokar et al., 1998; Marcum, 1998; Porter et al., 1994). In wheat, CMT or TTC correlated well with plant yield under heat stress. In ivy geraniums, both tests correlated well with plant width rather than GI and chlorosis. As ivy geraniums have a rambling growth habit, it is difficult to get accurate plant height measurements. Plant width was a more accurate indicator of plant growth. Chlorosis was scored on a 5 scale method. In the summer 2009, the temperatures were not high enough to cause significant foliar bleaching on most of the ivy geraniums, which may explain the low correlations between chlorosis and CMT and TTC tests. Another possibility is that chlorosis is a side-effect of heat stress that might not be caused by cell membrane change or change in cell respiration activity.

Between the CMT and TTC tests, the former had a greater correlation with the field trial data. This is similar to the finding of Fokar et al. (1998) in spring wheat where heat tolerance measured in terms of CMT was correlated with yields and that TTC was

positive but not significantly correlated with yields. They concluded that CMT was more predictive of plant heat tolerance than TTC. As the TTC test was significantly correlated with plant width in our research, TTC test, though not as predictive as CMT test, was also applicable in heat tolerance evaluation in ivy geraniums. The difference in the correlation of the two tests to plant width is unclear. It is possible that, compared with cell respiration, cell membrane thermostability was more seriously affected by heat stress. Further studies are needed to address this issue.

CMT and TTC tests can be employed to predict heat tolerance in ivy geraniums. Compared to whole plant screening technology, CMT and TTC tests are more time and cost efficient than field trials as they require a smaller sample size for the tests and can be done with fewer plants thereby reducing the selection period significantly. However, these tests are not perfect and cannot completely replace whole plant screening technology.

Table 4.1 Ivy geranium cultivars tested for thermal tolerance using cell membrane thermostability and triphenyl tetrazolium chloride tests.

Cultivar	Abbreviation
Acapulco Compact Cascade	AC
Beach	BE
Blizzard Subcompact Burgundy	BL
Caliente Deep Red	CD
Blizzard Red 09	BR
Taj Mahal	TA
Shiva	SH
Caliente Coral	CC
Freestyle Arctic Red	FR
Caliente Rose	CR
Lambada 09	LA

Table 4.2 Average growth index, average width, and average chlorosis ratings of 11 ivy geranium cultivars grown outside in a full sun location on a gravel nursery production yard at Mississippi State University from 15 May to 1 August 2009

Cultivar	Width (cm)	Growth Index[z] (cm^3)	Chlorosis[y]
Acapulco Compact Cascade	58.63 a[x]	47833 a	4.25
Beach	53.38 ab	31389 bcde	4.25
Blizzard Subcompact Burgundy	53.00 abc	41469 ab	4.25
Caliente Deep Red	50.25 abcd	38675 abc	4.5
Blizzard Red 09	47.25 bcde	30120 bcde	4.5
Taj Mahal	47.13 bcde	24963 cde	4
Shiva	47.00 bcde	29458 bcde	3
Caliente Coral	46.75 bcde	33454 abcd	4.75
Freestyle Artic Red	44.75 cde	20277 de	2.75
Caliente Rose	43.86 de	31879 bcd	5
Lambada 09	39.63 e	17332 e	3

(average temperatures were 31/19°C, d/n).
[z]Growth Index = 3.14 × ((average width/2) × (average width/2))^2 × height.
[y]Chlorosis is rated on a visual scale of 1 to 5 where a rating of 5 = no chlorosis, 4 = < 25% of the plant chlorotic, 3 = 50% of the plant chlorotic, 2 = 75% of the plant chlorotic, and 1 = 100% of the plant chlorotic.
[x]Means separation within columns using Duncan's Multiple Range test. Values with the same letters are not significantly different at $P < 0.05$.

Table 4.3 Correlations between cell membrane thermostability (CMT) test, triphenyl tetrazolium chloride (TTC) test and physical growth measurements of 11 ivy geraniums cultivars.

	Physical Growth Measurements			Test	
Tests	Growth Index[z]	Width	Chlorosis[y]	CMT	TTC
CMT	-0.35*[x]	-0.52**	0.01	--	-0.52**
TTC	0.31*	0.41**	0.12	-0.52**	--

CMT and TTC was conducted on plants exposed to 35°C temperatures for 24 h and growth measurement were from plants grown outside in a full sun location on a gravel nursery production yard at Mississippi State University from 15 May to 1 August 2009 (average temperatures were 31/19°C, d/n).
[z] Growth Index = 3.14 × ((average width/2) × (average width/2))^2 × height.
[y]Chlorosis rated on a visual scale of 1 to 5 where a rating of 5 = no chlorosis, 4 = < 25% of the plant chlorotic, 3 = 50% of the plant chlorotic, 2 = 75% of the plant chlorotic, and 1 = 100% of the plant chlorotic.
[x]* Significant $p < 0.05$, ** Significant $p < 0.01$

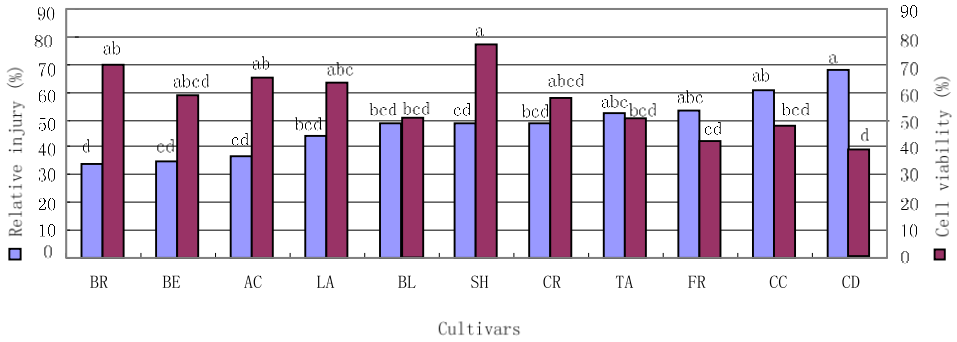

Figure 4.1 Cell Membrane Thermostability (CMT) relative injury values and Triphenyl Tetrazolium Chloride (TTC) cell viability tests of 11 ivy geranium cultivars subjected to heat stress to measure thermotolerance.

Plants were exposed to 35°C temperatures for 24 h prior to testing. BR = Blizzard Red '09, BE = Beach, AC = Acapulco Compact Cascade, LA = Lambada '09, BL= Blizzard Subcompact Burgundy, SH = Shiva, CR = Caliente Rose, TA = Taj Mahal, FR = Freestyle Artic Red, CC = Caliente Coral, CD = Caliente Deep Red. Means separation within CMT and TTC tests using Duncan's Multiple Range test. Values with the same letters are not significantly different at $P < 0.05$.

4.6 Literature Cited

Blum, A., N. Klueva and H.T. Nguyen. 2001. Wheat cellular thermotolerance is related to yield under heat stress. Euphytica 117: 117-123.

Chen, H.H., Z.Y. Shen and P.H. Li. 1982. Adaptiability of crop plants to high temperature stress. Crop Sci. 22: 719-725.

Dhanda, S.S. and R. Munjal. 2006. Inheritance of cellular thermotolerance in bread wheat. Plant Breeding 125: 557-564.

Dhir, R., R.L. Harkess and G. Bi. 2013. Response of ivy geranium ‘Beach' and ‘Butterfly' to heat stress. J. Amer. Soc. Hort. Sci. 138: 344–349.

Dhir, R., R.L. Harkess and G. Bi. 2011. Elevated air temperatures cause foliar bleaching of ivy geranium ‘Beach’ and ‘Butterfly’. HortScience 46: 411-415.

Duncan, D.R. and J.M. Widholm. 2004. Osmotic induced stimulation of the reduction of the viability dye 2,3,5-triphenyltetrazolium chloride by maize roots and callus cultures. J. Plant Physiol. 161: 397-403.

Fokar, M., H.T. Nguyen and A. Blum. 1998. Heat tolerance in spring wheat I. Estimation of cellular thermotolerance and its heritability. Euphytica 104: 1-8.

Gu, M. and M. Zhang. 2012. Heat Tolerance of Heucheras Using Laboratory-Based Methods. SNA research Conference. 57: 146-148

Jiang, Y. and B. Huang. 2000. Effects of drought or heat stress alone and in combination on Kentucky bluegrass. Crop Sci 40: 1358-1362.

Laken, G. 1949. The topographical tetrazolium method for determining the germination capacity of seeds. Plant Physiol. 24: 389-394.

Lu, Z., J. Chen, R.G. Percy, M.R. Sharifi, P.W. Rundel and E. Zeiger. 1996. Genetic variation in carbon isotope discrimination and its relation to stomatal conductance in pima cotton (*Gossypium barbadense*). Aust. J. Plant Physiol. 23: 127-132.

Marcum, K.B. 1998. Cell membrane thermostability and whole-plant heat tolerance of Kentucky bluegrass. Crop Sci. 38: 1214-1218.

Porter, D.R., H.T. Nguyen and J.J. Burk. 1994. Quantifying acquired thermal tolerance in winter wheat. Crop Sci 34: 1686-1689.

Martineau, J.R., J.E. Specht, J.H. Williams and C.Y. Sullivan. 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability. Crop Sci. 27: 579-585.

Reynolds, M.P., S. Nagarajan, M.A. Razzaue and O.A.A. Ageeb. 1997. Using canopy temperature depression to select for yield potential of wheat in heat-stressed environmental. Wheat Special Report. CIMMYT, Mexico.

Ruter, J.M. 1993. Foliar heat tolerance of two hybrid hollies. Hortscience 28: 650-652.

Saadalla, M.M., J.S. Quick and J.F. Shanahan. 1990. Heat tolerance in winter wheat: II. Membrane thermostability and field performance. Crop Sci. 30: 1248-1251.

Singh, P.H., P.V.V. Prasad, K. Sunita, S.N. Giri and K.R. Reddy. 2007. Influence of high temperature and breeding for heat tolerance in cotton: A review. Adv. Agron. 93: 313-385.

Sullivan, C.Y. 1979. Selecting for drought and heat resistance in grain sorghum. In: M.H. and S.R., editors, Stress physiology in crop plants. Wiley & Sons, New York. p. 263-281.

Wang, C.H. and D.M. Yeh. 2008. Heat tolerance and flowering-heat-delay sensitivity in relation to cell membrane thermostability in *Chrysanthemum*. J. Amer. Soc. Hort. Sci. 133: 754-759.

Yeh, D.M. and P.Y. Hsu. 2004. Heat tolerance in english ivy as measured by an electrolyte leakage technique. J. Hort. Sci. Biotechnol. 79: 298-302.

CHAPTER V

CONCLUSION

Changes in reactive oxygen species (ROS) and ROS scavenging enzymes were studied in ivy geranium. Changes in H_2O_2 content varies between heat tolerant and heat susceptible varieties. Heat tolerant varieties, such as 'Beach', tended to have more stable H_2O_2 content as well as more stable levels of ROS scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD). These findings indicate a stable ROS scavenging system may improve the heat tolerance of ivy geraniums. Although commonly thought to aid in heat tolerance, no correlation of active iron and heat tolerance was found. Iron content did not affect ivy geranium heat tolerance directly. Specifically, active iron content did not vary between leaf age or temperature treatment. The reports of iron improving heat tolerance in ivy geranium may result from chelated iron applications supplying sufficient iron to stimulate heat protection system enzyme synthesis. Cell membrane thermostability and cell respiration activity are both related to heat tolerance and Cell Membrane Themostability (CMT) and Triphenyl Tetrazolium Chloride (TTC) cell viability tests may be useful in evaluating heat tolerance in ivy geraniums. Of the two tests, CMT had a greater correlation with the field trial data than TTC. Although these tests are more time and cost efficient than field trials, they have limitations and cannot completely replace field trials. Use of laboratory tests, such as CMT and TTC test, may significantly increase the efficiency of the variety selection

process. This research supports that the ROS scavenging system and cell membrane stability play an important role in ivy geranium heat tolerance mechanisms.